Validation of the anti-viral and sterilization effects of silver ion water produced from a silver ion water generator on pathogens originated from the chicken

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Submission

Addressed to CNL.
This report is submitted as the final report for the “validation of the anti-viral and sterilization effects of silver ion water produced from a silver ion water generator on pathogens originated from the chicken” project.

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Summary of Results

1. Title

Validation of the anti-viral and sterilization effects of silver ion water produced from a silver ion water generator on pathogens originated from the chicken.

2. Objective

To measure the anti-viral and sterilization effects of active silver ion water produced from a silver ion water generator on H9N2 low pathogenic avian influenza virus (LPAI) and two types of salmonella (salmonella enteritidis: SE and salmonella gallinarum: SG).

3. Contents and scope of test

The validation test was conducted as follows:

○ Anti-viral effect on LPAI virus

- The raw material (active silver ion water) in respective concentrations obtained from a silver ion water generator was mixed with the culture solution of LPAI and inoculated to specific pathogen free (SPF) eggs at different time points for up to 24 hr. All experiments were repeated twice, and the final virus levels (EID 50) were measured by the Reed and Muench method.

○ Effect on salmonella

- The raw material (active silver ion water) in respective concentrations obtained from a silver ion water generator was mixed with the culture solutions of two types of salmonella, i.e., SE and SG, applied to a BSA medium, cultured for 2 days and the sterilization effect was measured. All experiments were repeated twice.

4. Summary of test results

○ The anti-viral efficacy of active silver ion water on avian influenza virus was investigated using SPF egg inoculations. The results showed that the experimental group where samples containing active silver ion water at high concentrations (2, 5, and 10 ppm) were mixed with LPAI virus and reacted for 24 hr had statistically significant anti-viral effect compared to negative control.

○ The inhibitory effect of active silver ion water on salmonella gallinarum, one of major pathogens that cause chicken farms in Korea financial damages, and salmonella enteritidis that is a common cause of food poisoning was measured. Evident sterilization effects were observed from all experimental groups reacted with active silver ion water at low concentration for longer than 30 min.

Contents

1. Overview

The anti-viral or sterilization effect of the raw material produced using a silver ion water generator (hereinafter referred to as “active silver ion water”) was validated. The anti-viral effect was measured against low pathogenic avian influenza (LPAI H9N2) commonly found in the Korean poultry industry using SPF egg inoculations. The experiment was repeated twice to strengthen reliability. The sterilization effect was measured against, among many serum types of salmonella, salmonella enteritidis (SE) that has importance for public health and salmonella gallinarum (SG) that causes the nation’s poultry industry direct financial damages. All experiments were repeated twice.

2. Anti-viral effect of active silver ion water on LPAI virus (1st experiment at low concentration)

A. Objectives

To validate the anti-viral effect of low-concentration active silver ion water produced from a silver ion water generator on H9N2 LPAI virus.

B. Materials and methods

1) Sentinel animal

Specific pathogen free (SPF) eggs were imported from SPAFAS in the USA and incubated in an incubator in the Laboratory of Avian Diseases of the College of Veterinary Medicine at Chungbuk National University (Yoonsun Incubator, Suwon). The sample was inoculated at 11 days old.

2) Virus and culture

A/CK/ANYANG/MS96 (H9N2) AI virus that was separated and characterized in Korea was proliferated in SPF eggs and expanded to 10^9.3 EID50/ml using the Reed and Muench method and used for the experiment.

3) Number of silver ions per concentration and mixing with virus culture solutions

The silver ion water generator was set in four stages (0.25 ppm, 0.5 ppm, 1 ppm, and 2 ppm) and produced active silver ion water was used as the test solution. For each concentration, 9.9 ml of the test solution was mixed with 0.1 ml of LPAI virus culture solution.

The solution was diluted by 10^3 through to 10^8 immediately after mixing and after being reacted at room temperature for 30 min, 1 hr, 2, hr, 6 hr, and 24 hr, respectively, and 5 samples per concentration were inoculated to SPF eggs. After inoculations, the eggs were cultured at 37℃ for 2 days and chilled for more than 4 hr at refrigerating temperature and the egg's allantoic fluids were collected. For the collected allantonic fluids, virus positivity was determined by plate hemagglutination test using 5% chicken red cells. For virus levels after hemagglutination, EID50/ml was measured using the Reed and Muench method.

C. Results and discussions

Table 1. Anti-viral effect of silver ion water per concentration on avian influenza virus

Concentration of silver ion (ppm)	Incubation time (hr)	EID50/ml (log_{10} value)[b]		Average	p value
		1	2		
0	0	8.7	91.	8.9	
	0.5	8.8	8.4	8.6	
	2	8.7	8.6	8.65	
	6	9.5	8.6	9.05	
	24	8.6	8.6	8.6	
0.25	0	9.2	9.4	9.3	0.107768
	0.5	8.8	9.3	9.05	0.147533
	2	8.6	8.2	8.4	0.174528
	6	8.8	9.2	9	0.464194
	24	8.6	8.5	8.55	0.211325
0.5	0	9.4	9.3	9.35	0.115825
	0.5	9.6	8.8	9.2	0.155876
	2	8.8	8.6	8.7	0.349244
	6	8.6	9.4	9	0.470689
	24	8.5	8.8	8.65	0.385292
1	0	8.6	8.6	8.6	0.136197
	0.5	8.3	9.2	9.25	0.394721
	2	8.5	9.4	8.95	0.278768
	6	8.4	8.6	8.5	0.177584
	24	7.6	8.5	8.05	0.173059
2	0	8.6	9.5	9.05	0.394721
	0.5	8.5	8.6	8.55	0.415485
	2	8.7	8.8	8.75	0.146447
	6	8.5	8.8	8.65	0.243926
	24	7.5	7.8	7.65	0.012018*

* Statistically significant anti-viral effect compared to negative control ($p < 0.05$)

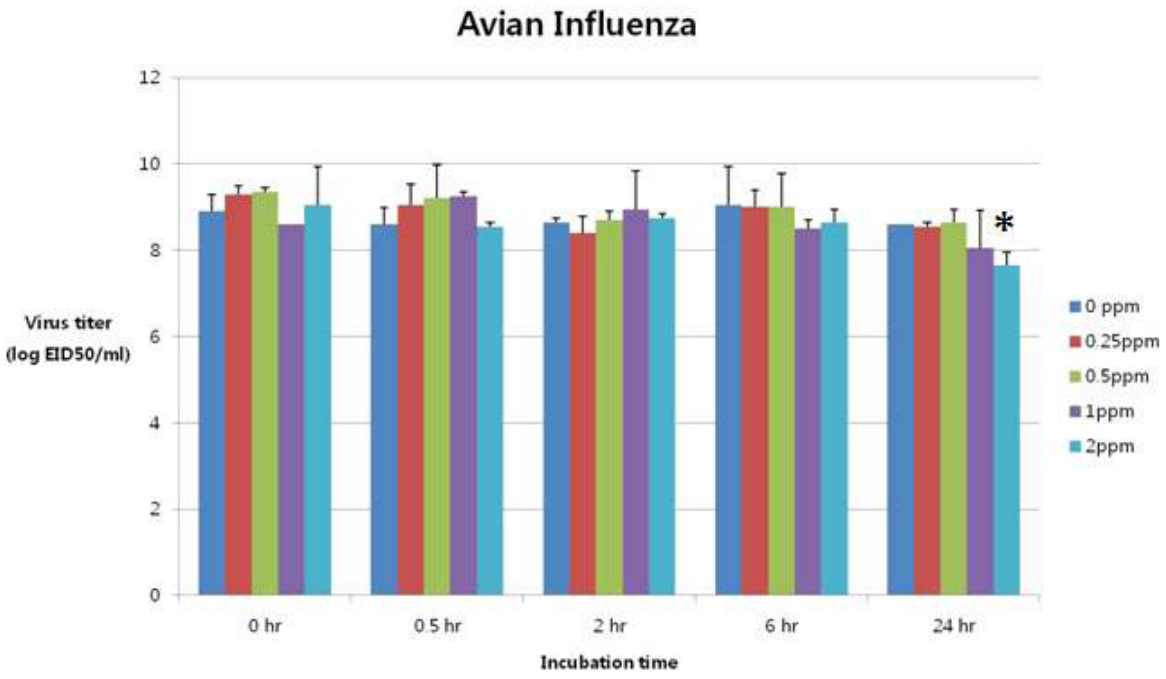

Figure 1. Anti-viral effect of silver ion water per concentration on avian influenza virus
Error bar: 95% confidence interval
* Statistically significant anti-viral effect compared to negative control ($p < 0.05$)

Neutralization tests by different concentrations of active silver ion water against H9N2 LPAI were conducted, and the results are summarized in Table 1 and Figure 1. For anti-viral effects at each concentration, statistically significant anti-viral effects compared to negative control were observed only when treated with 2 ppm active silver ion water for 24 hr or longer. Accordingly, additional experiments were conducted to measure anti-viral effects at concentrations higher than that of active silver ion water used in this experiment.

3. Anti-viral effect of active silver ion water on LPAI virus (2nd experiment at high concentration)

A. Objectives

To validate the anti-viral effect of high-concentration active silver ion water produced from a silver ion water generator on H9N2 LPAI virus.

B. Materials and methods

1) Sentinel animal

Specific pathogen free (SPF) eggs were imported from SPAFAS in the USA and incubated in an incubator in the Laboratory of Avian Diseases of the College of Veterinary Medicine at Chungbuk National University (Yoonsun Incubator, Suwon). The sample was inoculated at 11 days old.

Virus and culture

A/CK/ANYANG/MS96 (H9N2) AI virus that was separated and characterized in Korea was proliferated in SPF eggs and expanded to 10^9.3 EID50/ml using the Reed and Muench method and used for the experiment.

3) Number of silver ions per concentration and mixing with virus culture solutions

The silver ion water generator was set in three stages (0 ppm, 5 ppm, and 10 ppm) and produced active silver ion water was used as the test solution. For each concentration, 9.9 ml of the test solution was mixed with 0.1 ml of LPAI virus culture solution. The solution was diluted by 10^3 through to 10^8 immediately after mixing and after being reacted at room temperature for 30 min, 1 hr, 2, hr, 6 hr, and 24 hr, respectively, and 5 samples per concentration were inoculated to SPF eggs. After inoculations, the eggs were cultured at 37℃ for 2 days and chilled for more than 4 hr at refrigerating temperature and the egg's allantoic fluids were collected. For the collected allantonic fluids, virus positivity was determined by plate hemagglutination test using 5% chicken red cells. For virus levels after hemagglutination, EID50/ml was measured using the Reed and Muench method.

C. Results and discussions

Table 2. Anti-viral effect of high concentration silver ion water on avian influenza virus

Concentration of silver ion (ppm)	Incubation time (hr)	EID50/ml (log_{10} value)[b]		Average	p value
		1	2		
0	0	9.6	9.8	9.75	
	0.5	9.6	9.6	9.6	
	2	9.6	9.8	9.7	
	6	9.8	9.6	9.7	
	24	8.2	8.2	8.2	
5	0	9.5	9.4	9.45	0.077423
	0.5	8.7	9.5	9.1	0.168867
	2	9.3	8.7	9	0.07865
	6	9.4	8.5	8.95	0.122634
	24	0	0	0	<0.00001*
10	0	9.4	9.2	9.3	0.052786
	0.5	9.5	8.4	8.95	0.179379
	2	7.7	9.2	8.45	0.120164
	6	8.7	8.8	8.75	0.006785*
	24	0	0	0	<0.00001*

* Statistically significant anti-viral effect compared to negative control ($p < 0.05$)

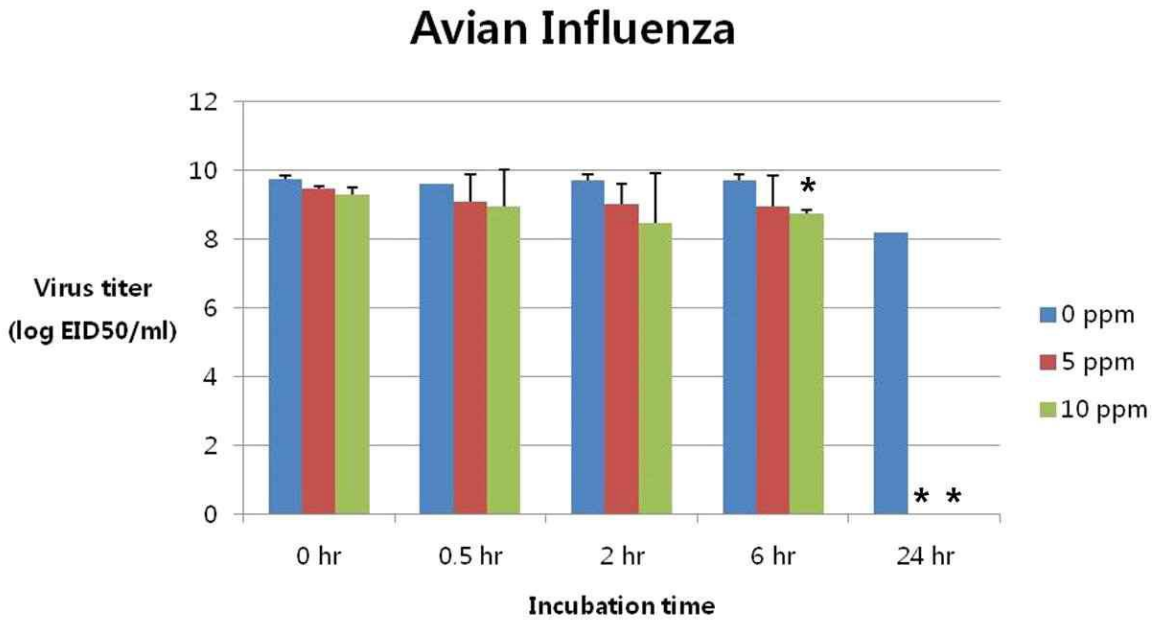

Figure 2. Anti-viral effect of high concentration silver ion water on avian influenza virus
Error bar: 95% confidence interval
* Statistically significant anti-viral effect compared to negative control ($p < 0.05$)

Neutralization tests by different concentrations of active silver ion water against H9N2 LPAI were conducted, and the results are summarized in Table 2 and Figure 2. For anti-viral effects at each concentration, statistically significant anti-viral effects compared to negative control were observed when treated with 5 ppm silver ion water for 24 hr and when treated with 10 ppm silver ion water for 6 hr or longer. In particular, treatment with high concentration silver ion water for 24 hr or longer resulted in the total annihilation of LPAI virus.

4. Inhibitory effect of active silver ion water on salmonella enteritidis

A. Objectives

To measure the sterilization effect of active silver ion water produced from a silver ion water generator on salmonella enteritidis that is a common cause of food poisoning.

B. Materials and methods

1) Bacterial culture

Salmonella enteritidis (SE) that was separated from a chicken farm in Korean and serologically characterized was cultured in tryptic soy broth (TSB) at 37℃ for 24 hr, followed by centrifugation at 4000g for 20 min and washing and resuspension with 0.1 peptone water (pH 7.1). It was used as the type strain.

2) Silver ion water per concentration, mixing with bacterial culture solution, and culture

To allow for the measurement of bacterial colonies in solid media, the liquid culture solution was diluted by 10^5. 0.1 ml of the diluted solution and 9.9 ml of active silver ion water at each concentration were applied to a tryptic soy agar (TSA) medium and cultured at 37℃ for 48 hr.

3) Colony counting

All experiments were repeated twice. The calculation of bacterial colonies cultured in TSA solid media were conducted by a blind test

Table 3. Sterilization effect of silver ion water on salmonella enteritidis

Concentration of silver ion (ppm)	Incubation time (hr)	Number of colonies		Average	p value
		1	2		
0	0	388	402	395	
	0.5	168	131	149.5	
	2	30	16	23	
	6	3	4	3.5	
	24	0	0	0	
0.25	0	379	312	345.5	0.142494
	0.5	19	21	20	0.00993*
	2	0	0	0	0.04703*
	6	0	2	1	0.077423
	24	0	0	0	NA**
0.5	0	259	340	349.5	0.07289
	0.5	38	21	29.5	0.013799*
	2	0	0	0	0.040734*
	6	0	0	0	0.009902*
	24	0	0	0	NA**
1	0	208	409	308.5	0.240514
	0.5	33	33	33	0.012151*
	2	0	0	0	0.040734*
	6	0	0	0	0.009902*
	24	0	0	0	NA**
2	0	281	384	332.5	0.176105
	0.5	7	6	6.5	0.00817*
	2	0	0	0	0.040734*
	6	0	0	0	0.009902*
	24	0	0	0	NA**

* Statistically significant sterilization effect compared to negative control ($p < 0.05$)

** Not applicable.

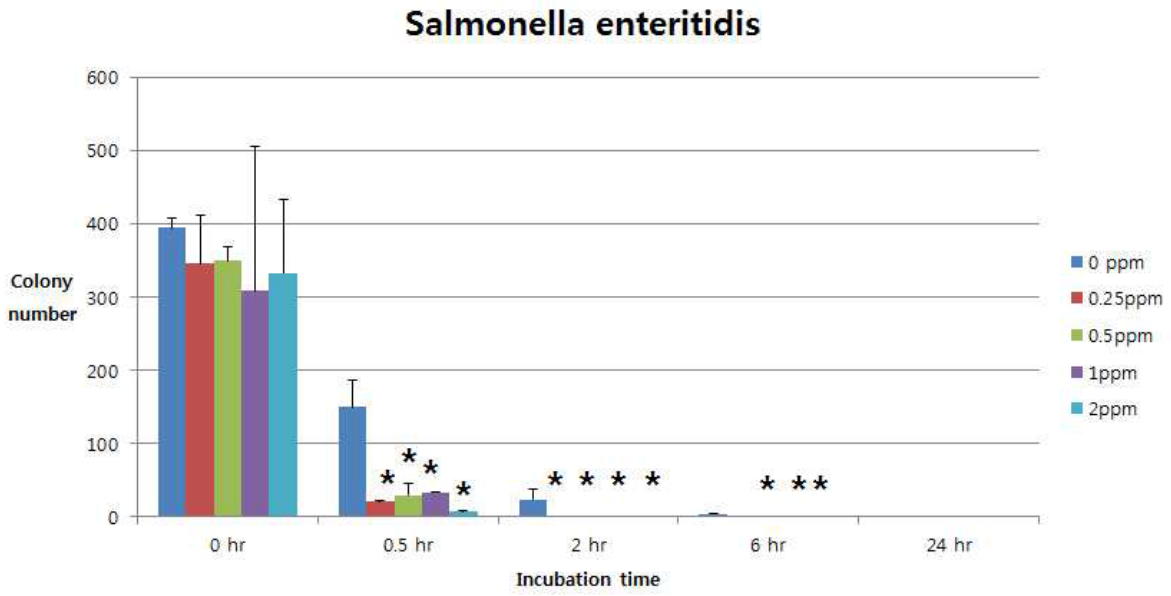

Figure 3. Sterilization effect of silver ion water on salmonella enteritidis
Error bar: 95% confidence interval
* Statistically significant sterilization effect compared to negative control ($p < 0.05$)

Neutralization tests by different concentrations of active silver ion water against salmonella enteritidis that is a common cause of food poisoning in Korea were conducted, and the results are summarized in Table 3 and Figure 3. For sterilization effects at each concentration, statistically significant sterilization effects were observed when silver ion water at 0.25 ppm or higher concentrations was treated to cultured bacteria for 30 min or longer.

5. Inhibitory effect of active silver ion water on salmonella gallinarum

A. Objectives

To measure the sterilization effect of active silver ion water produced from a silver ion water generator on salmonella enteritidis.

B. Materials and methods

1) Bacterial culture

Salmonella enteritidis (SE) that was separated from a chicken farm in Korean and serologically characterized was cultured in tryptic soy broth (TSB) at 37℃ for 24 hr, followed by centrifugation at 4000g for 20 min and washing and resuspension with 0.1 peptone water (pH 7.1). It was used as the type strain.

2) Silver ion water per concentration, mixing with bacterial culture solution, and culture

To allow for the measurement of bacterial colonies in solid media, the liquid culture solution was diluted by 10^5. 0.1 ml of the diluted solution and 9.9 ml of active silver ion water at each concentration were applied to a tryptic soy agar (TSA) medium and cultured at 37℃ for 48 hr.

3) Colony counting

All experiments were repeated twice. The calculation of bacterial colonies cultured in TSA solid media were conducted by a blind test

Table 4. Sterilization effect of silver ion water on salmonella gallinarum

Concentration of silver ion (ppm)	Incubation time (hr)	Number of colonies		Average	p value
		1	2		
0	0	324	245	284.5	
	0.5	43	50	46.5	
	2	14	12	13	
	6	0	0	0	
	24	0	0	0	
0.25	0	216	200	208	0.099048
	0.5	2	0	1	0.00317*
	2	0	0	0	0.002933*
	6	0	0	0	NA**
	24	0	0	0	NA**
0.5	0	218	273	245.5	0.251426
	0.5	0	4	2	0.004053*
	2	0	0	0	0.002933*
	6	0	0	0	NA**
	24	0	0	0	NA**
1	0	221	141	181	0.103477
	0.5	1	1	1	0.002933*
	2	0	0	0	0.002933*
	6	0	0	0	NA**
	24	0	0	0	NA**
2	0	80	129	104.5	0.030338*
	0.5	0	0	0	0.002809*
	2	0	0	0	0.002933*
	6	0	0	0	NA**
	24	0	0	0	NA**

* Statistically significant sterilization effect compared to negative control ($p < 0.05$)

** Not applicable.

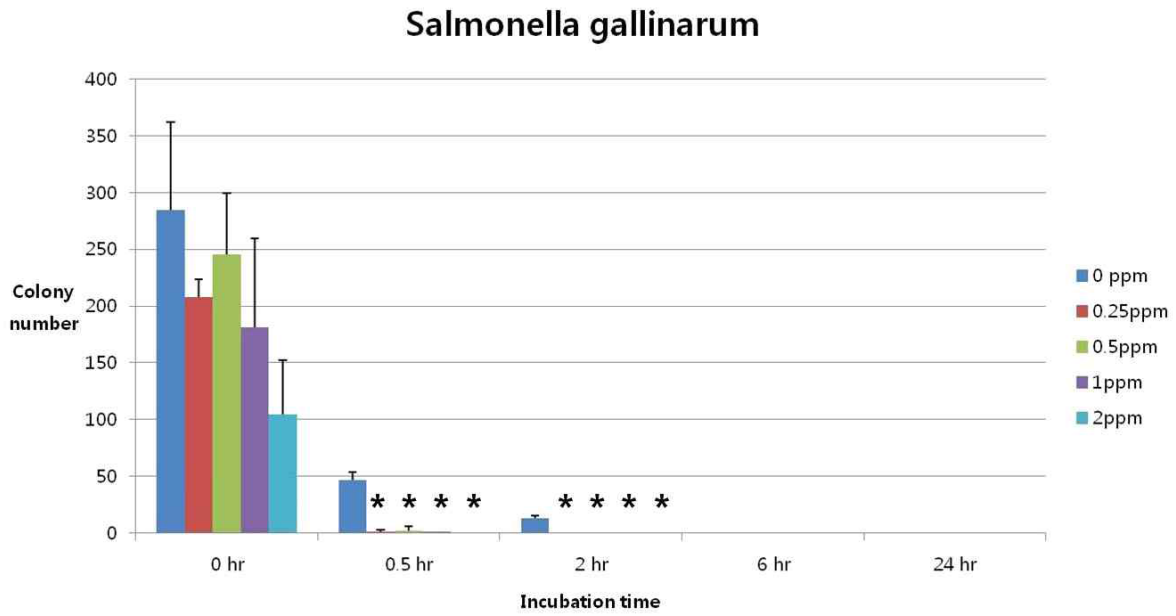

Figure 4. Sterilization effect of silver ion water on salmonella gallinarum
Error bar: 95% confidence interval
* Statistically significant sterilization effect compared to negative control ($p < 0.05$)

Sterilization tests by different concentrations of active silver ion water against salmonella gallinarum that causes the chicken farms in Korea financial damages were conducted, and the results are summarized in Table 4 and Figure 4. For sterilization effects at each concentration, statistically significant sterilization effects were observed when silver ion water at 0.25 ppm or higher concentrations was treated to cultured bacteria for 30 min or longer. In particular, 2 ppm active silver ion water showed very fast sterilization action from 30 min.

6. Final discussion over research project

In this test the anti-viral and sterilization effects of active silver ion water produced from a silver ion water generator on pathogens originated from the chicken. For H9N2 LPAI used for this test, statistically significant anti-viral effects were observed when treated with silver ion water at relatively higher concentrations (2 ppm, 5 ppm, and 10 ppm) for 24 hr or longer. But at 10 ppm, anti-viral effects were observed from 6 hr. This result implies that for deactivation of virus, at least 6 hr contact with active silver ion water is required at a high concentration (10 ppm) and at least 24 hr contact is required at normal concentrations between 2 ppm and 10 ppm.

Sterilization effects on two types of salmonella, i.e., salmonella enteritidis and salmonella gallinarum that cause huge financial damages to chicken farms and food poisoning to humans were measured. The results showed that at least 30 min treatment from low (0.25 ppm) to high (2 ppm) concentrations was enough to obtain efficient sterilization effects.

From the test results, it is thought that in laboratory environments active silver ion water treatment will have efficient anti-viral and sterilization effects at 10 ppm or higher on avian influenza and at 0.25 ppm or higher on salmonella. However, in the actual practice of the chicken farm industry there are various farm environments, therefore various factors in various farms should be taken into account to produce anti-viral or sterilization effects from this test's raw material, that is, active silver ion water.